

significantly fluorescent when incubated either with the antibody against fibronectin or galactocerebrosides. Since it had been reported that the GFAP immunoreactivity could not be detected in 13-day-old mouse embryos [13], we decided to follow the time course of appearance of this marker in culture. We found out that vimentin- and GFAP-positive cells were already present after 1 day in culture; moreover, vimentin did not seem to diminish with time. In order to further test this later point, newborn cells were plated in the presence of serum. After 8 days in vitro most cells were unable to respond to an antibody against neurofilament, all cells were vimentin-positive among which 30–50% were GFAP-positive, the only difference from younger cells being the more mature aspects of the astrocytes. Again, no galactocerebrosides of fibronectin-containing cells were seen.

In conclusion, we can say that in CCDM 95% of the cells are neuronal in nature. The other cells develop very well in the presence of serum; most of them, if not all, are vimentin-positive. Among these vimentin-positive cells 30–50% are GFAP-positive and can be considered as astroblasts. These astrocytes are present very early (1 day after the plating of 13-day-old embryos) and contain vimentin at all times tested. No fibroblasts or galactocerebroside-containing cells could be detected. The nature of the cells positive for vimentin, but not stained by any other intermediate filament or specific marker is still undetermined.

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Sizes of chromosome loops and hnRNA molecules in oocytes of amphibia of different genome sizes

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Summary. The lengths of lampbrush chromosome loops and their transcription units show a positive cor-

relation with genome size in oocytes of amphibia with different C values. However, there is no such correlation with contour lengths of hnRNA molecules isolated from these oocytes. These results indicate that more DNA sequences are transcribed in amphibia of higher C value, but that processing of RNA transcripts occurs while they are still attached to the chromosomes as nascent ribonucleoprotein fibrils.

The functional significance of the possession of apparently excessive amounts of nuclear DNA by higher eukaryotes remains unclear. The phenomenon of large variation in genome size (C value) within classes of phenotypically similar organisms is particularly apparent in the amphibia, a class which contains genera having a range of 30-fold difference in C value (table 1). Yet it has been demonstrated that in oocytes of *Xenopus* and *Triturus*, organisms whose C values differ by a factor of 7, both populations of poly(A)⁺ messenger RNA (mRNA) molecules have similar sequence complexities and are preferentially transcribed from single-copy DNA sequences [1]. One crucial aspect of this "C-value paradox" concerns the extent to which DNA is transcribed into RNA. Is there generally more extensive transcription in higher C value organisms and, if so, can this extra transcription be accounted for by the presence of longer non-coding flanking and intervening sequences in longer primary transcripts and heterogeneous nuclear RNA (hnRNA) molecules? In a previous study, the size of RNA transcripts in cell lines of the diptera *Drosophila* and *Aedes* have been compared [2]. *Aedes* has a 5–6-fold larger genome than does *Drosophila* and produces hnRNA which is on average 2–2.5-fold longer. In the report we compare the lengths of chromosome loops and transcriptional units with the contour lengths of hnRNA molecules from the germinal vesicles of amphibia with a much wider range of C values.

Materials and Methods

Nuclei were manually isolated from previtellogenic oocytes of *Xenopus laevis*, *Triturus cristatus carni-fex*, *Amphiuma means* and *Necturus maculosus* in Tris-buffered "3:1" solution (75 mM KCl, 25 mM NaCl, 10 mM Tris-HCl, pH 7.2) and transferred immediately into ice-cold 70% ethanol. After collecting 50–200 nuclei, they were pelleted by centrifugation at 200 g for 2 min. The drained pellet was raised in 0.2 ml of a solution containing 1% sarkosyl NL-97 (Ciba-Geigy), 50 mM Tris-HCl, pH 8.4, 20 mM EDTA and 0.5 mg/ml proteinase K (Boehringer, Mannheim; pre-digested at 37°C for 30 min). After incubation at 18°C for 6–12 h, RNA was extracted by a modification of the technique of Glisin et al. [3]. Solid CsCl was added to 1 mg/ml and the solution was layered over 0.3 ml of 5.7 M CsCl, 0.1 M EDTA in a 1 ml centrifuge tube. The RNA was pelleted through the CsCl cushion by centrifugation at 38000 rpm for 16 h at 25°C in an MSE 3×5 ml swing-out rotor equipped with adaptors for the small tubes. Then the bottom of the tubes was carefully cut off, the pellet drained and the RNA resuspended in 5–50 µl of TE (10 mM Tris-HCl, pH 7.2, 1 mM EDTA). The spreading solution contained 70–90% formamide, 4 M urea, 40 mM Tris-HCl, pH 8.5, 1 mM EDTA and 50 µg/ml cytochrome c [4]. In several experiments the RNA was heated to 60°C for 1 min to remove secondary structures prior to adding cytochrome c. Spreading was on distilled water as hypophase. RNA molecules were collected on paralodion-coated grids and processed for electron microscopy as described [4–6]. Contour lengths were determined by tracing the molecules on calibrated prints using a digitizer tablet. For controls, larger vitellogenic oocytes of *Xenopus laevis* and *Triturus cristatus* were used to extract nuclear RNAs. Our procedures used did allow perfect demonstration of intact pre-rRNA molecules (cf ref. [5]), indicating that the isolation and microscopy procedures employed allow the preservation of intact RNA molecules exceeding 2.5 µm in lengths.

Lampbrush chromosomes were prepared in the 3:1 medium adjusted to 0.1 mM CaCl₂ as described previously [7] and photographed under phase-contrast optics.

Results and Discussion

On different criteria the extent of genome transcription in amphibian oocytes appears to approximate to a fixed percentage. Molecular hybridization experiments indicate that about 4% of the genomic DNA of *Triturus* is transcribed into oocyte hnRNA [8] and a similar value can be estimated for *Xenopus* oocyte transcription (for discussion, see [9]). A more direct approach is to compare the sizes of the lateral lampbrush chromosome loops which are the

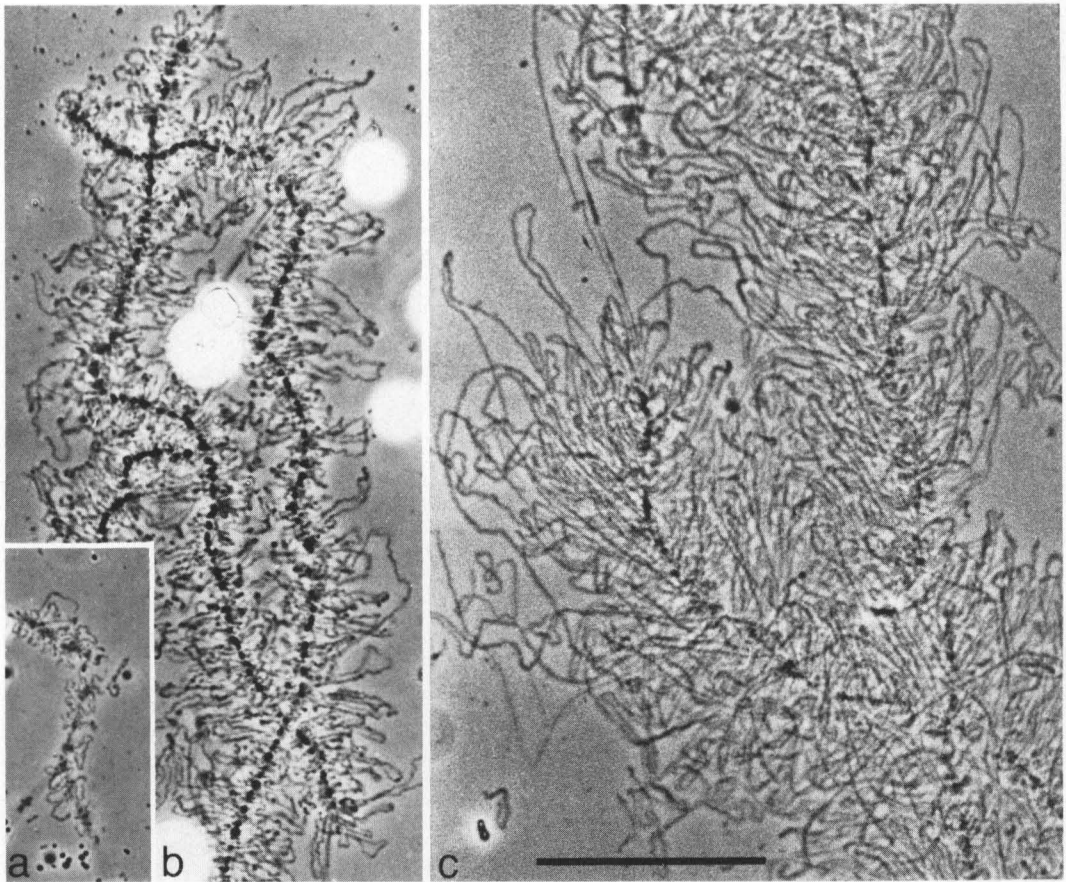


Fig. 1. Relative dimensions of lateral loops in lampbrush chromosomes isolated from oocytes of different C-value amphibia, as seen in phase contrast. Prepara-

tions from (a) *Xenopus laevis*; (b) *Triturus cristatus*, and (c) *Necturus maculosus* are shown at the same magnification. Bar, 50 μm .

manifestation of transcriptional activity. Fig. 1 shows the relative dimensions of lampbrush chromosome structures derived from germinal vesicles of *Xenopus*, *Triturus* and *Necturus*. The chromosomes have been isolated from oocytes which were at the same (early vitellogenic) stage of oogenesis. It is obvious that there are striking differences in the sizes of the lateral loops between different amphibian species.

The majority of the loops in *Triturus* fall within the size range from 30 to 50 μm , but are much smaller in *Xenopus* (5–10 μm)

and much longer in *Necturus* (mean >100 μm). Furthermore the lateral loops of *Amphiuma* are similar in size to those of *Necturus* and in general there is a good positive correlation between genome size and lateral loop length in a number of different amphibia (see also [10]). From the number of lateral loops per chromosome set and their average sizes it can be calculated that the percentage of genomic DNA that is contained in the transcribing loops is relatively constant at 5–10% for the various amphibia. The observations pertaining to the

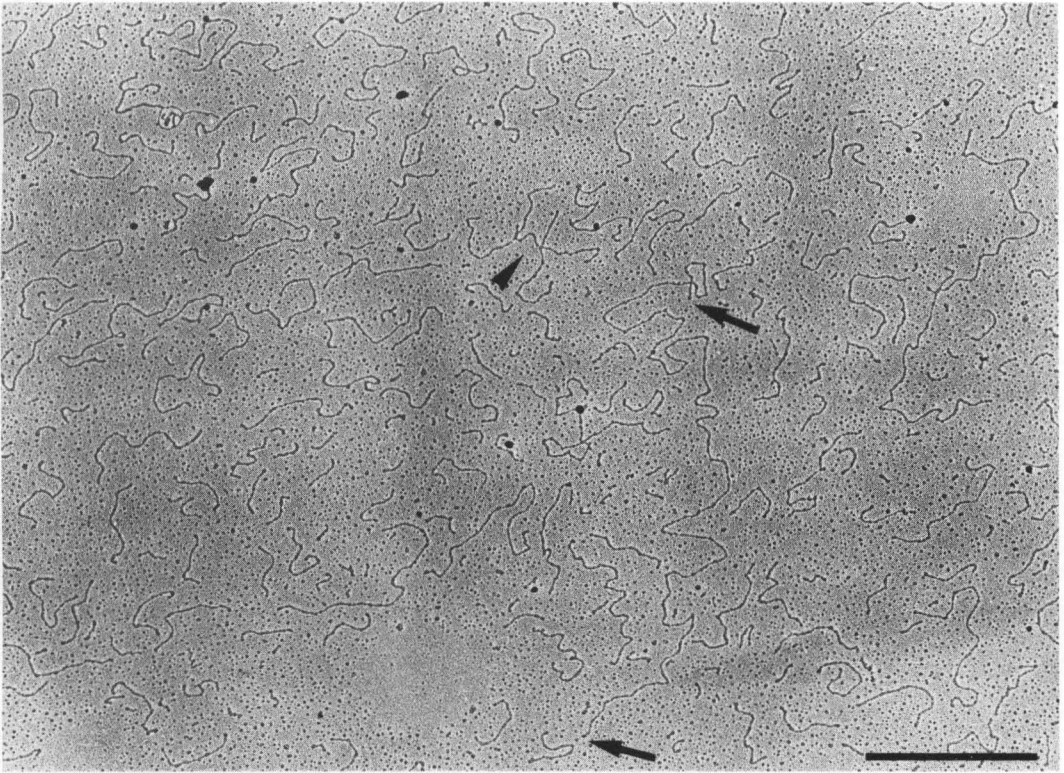


Fig. 2. hnRNA molecules extracted from previtellogenic *Amphiuma* oocyte nuclei and spread for electron microscopy. Some foldback structures and duplex regions between individual RNA molecules (arrow-

head) are visible. The long molecule denoted by the two arrows has a contour length of $6.8 \mu\text{m}$. Bar, $1 \mu\text{m}$.

relative lengths of loops in lampbrush chromosomes also appear to hold for transcriptional units seen in electron-microscopic spread preparations, although for technical reasons it is more difficult to carry out a statistical study on spread chromatin preparations. This is especially the case for the amphibian species with large genome sizes: here it is almost impossible to measure the length of transcriptional units due to their enormous sizes. Since, however, for many loci, lateral loop length is proportional to the length of transcriptional units (although multiple transcriptional units can exist within a single loop; for discussion, see [11])

we might expect to find correspondingly longer hnRNA molecules in the germinal vesicles of higher C-value animals. This expectation is based on the observation that the frequency of the occurrence of multiple transcriptional units along individual loops is not higher in species with higher DNA contents, as judged from light microscopic preparations. Thus, the increase of loop length cannot be attributed to a multiplication and tandem arrangement of transcriptional units along individual loops, but rather seems to reflect longer transcriptional units.

RNA molecules were extracted from

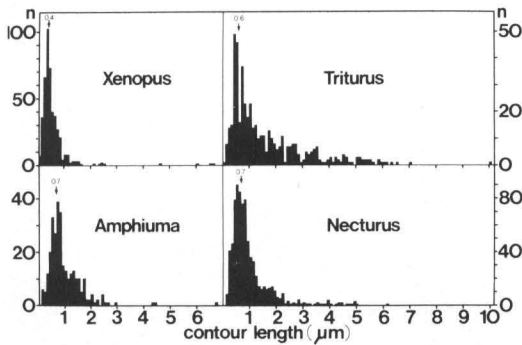


Fig. 3. Contour length analysis of hnRNA molecules from oocytes of different C-value amphibia. One micrometer of contour length corresponds to a MW of about one million.

nuclei of previtellogenic oocytes, because at this stage there is negligible transcription of rRNA genes, thus allowing an analysis of pure hnRNA molecules. The RNA was surface-spread under denaturing conditions and examined by electron microscopy. A contour length analysis was made of RNA preparations from several different amphibia (a preparation from *Amphiuma* is shown in fig. 2) and the results were plotted as histograms. It is apparent from the histograms of fig. 3 that (i) the majority of the hnRNA molecules are relatively short; and (ii) their length distribution is strikingly similar in the different amphibian species studied and does not reveal a correlation with the sizes of the lateral loops. All histograms show a distinct peak of RNA molecules of sizes centered around 0.4–0.7 μm (corresponding to the length of poly(A)⁺ RNA molecules extracted from the ooplasm; Scheer & Sommerville, unpublished). The distributions are skewed to the right, due to the presence of longer RNA molecules of up to 10 μm in length (corresponding to a MW of about 10×10^6). Molecules longer than 10 μm were not observed although, e.g., in *Triturus* the average loop length is 30–50 μm and most tran-

scriptional units fall within the size range of 3–30 μm with expected MWs of their primary transcripts of $3\text{--}30 \times 10^6$ (ca 3–30 μm contour length of the surface-spread molecules). Furthermore, in the high C-value organisms *Amphiuma* and *Necturus*, no molecules are found exceeding 7 μm in length, although many of the lateral loops in the chromosomes of these organisms are about 100 μm long. Therefore, in comparing hnRNA length with C value it can be seen that, although the lengths of hnRNA molecules from *Triturus* are generally longer than those from *Xenopus*, there is no increasing trend in hnRNA size when the higher C-value organisms are considered.

These results are in agreement with the findings obtained by other authors using cultured insect cells [2] in that we also observe a positive correlation between the lengths of transcriptional units and genome sizes. In contrast to these authors, however, we do not observe a corresponding increase in the size of the nuclear RNA molecules. Possible explanations of these results are that hnRNA molecules are longer in vivo and are degraded during extraction and mounting for electron microscopy or that the RNA transcripts are rapidly processed to give nuclear populations of shorter length RNA molecules. The first

Table 1. C values of different amphibia

Organism	C value	
	pg/haploid genome ^a	Ratio
<i>Xenopus laevis</i>	3.1	1
<i>Triturus cristatus</i>	23	7.4
<i>Amphiuma means</i>	65	21.0
<i>Necturus maculosus</i>	78	25.1

^a For reference, see [17].

possibility is made less likely by the observation that when RNA molecules are prepared under identical conditions from vitellogenic oocytes, rRNA precursors can be recognised by virtue of their secondary structure and are always of the length expected of intact molecules (2.6–2.8 μm ; see also [5]). The second possibility, that primary transcripts are rapidly cleaved by natural processing steps, raises the question of whether RNA is cleaved prior to its release from chromatin. Several lines of evidence suggest that this may indeed be the case: e.g., a substantial proportion (as much as 50%) of radioactively labelled nuclear RNA extracted from previtellogenic oocytes is derived from the nascent RNP transcripts of lampbrush chromosomes [12] and therefore the RNA molecules of nascent transcripts should be well represented in the total nuclear RNA population. That molecules as long as the average length of transcriptional units are not found would suggest that the RNA molecules that exist within the RNP transcripts are extensively nicked while the transcripts are attached to the chromatin, and that the integrity of RNP fibrillar structures is maintained by virtue of protein components. However, even protein linkage may not be sufficient to maintain intact RNP fibril lengths proportional to the length of chromatin transcribed, for discontinuities are occasionally found in the gradient of transcript lengths within transcriptional units (see [11, 13–15]) and in lampbrush chromosome preparations specific RNA sequences can be lost from lateral loops part way through a region of continuous transcription [16]. Therefore it seems reasonable to suggest that much of the processing of nuclear RNA in oocytes occurs while the RNA sequences are still attached to the chromatin in the form of nascent RNP fibres. Since the length of

RNA molecules found in germinal vesicles of the high C-value organisms *Amphiuma* and *Necturus* are no longer than molecules from organisms of much lower C value, it would appear that the non-coding sequences which constitute by far the most abundant component of the long primary transcripts in these genera exist either as continuous stretches of about 3×10^5 or more nucleotides which are rapidly degraded, or as multiple intervening sequences which are excised during RNA splicing. The analysis of the precise organization of coding sequences within exceedingly long transcriptional units is now technically feasible [18] and awaits further investigation.

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Cell surface and clonal proliferative property of aging human diploid fibroblasts

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Summary. We have described previously how concanavalin A (conA)-coated red blood cell (RBC) adsorption to human diploid fibroblasts could serve as a marker of the in vitro aging of these cells. Since the heterogeneity in RBC adsorption and the proliferative property of young and old cell populations was observed, the correlation of this cell surface property with the proliferative behavior of individual clones was examined as a function of cell age. The RBC adsorption capacity of cells in both large and small colonies changed with the aging of the parent cell populations; the cells from early passage populations did not adsorb RBCs, but those from late passage populations adsorbed them well. Thus, the amount of RBC adsorption was not a function of colony size, but was related to the age of the culture.

Human diploid fibroblasts have a limited doubling potential in vitro [1], and an increasing heterogeneity with their aging has been known with respect to their proliferative behavior and size of individual cells or colonies [2-6]. Thus, cell size, [³H]thymidine labelling index and colony size distribution have been used as markers of cellular aging in these cells [7-9]. However, it is questionable whether these parameters are directly related to the remaining doubling potential at the individual cell level.

For example, the recultivation of fractionated cells of varying size did not yield subpopulations of cells with different long-term proliferative potentials [10, 11]. A marker which reflects the remaining doubling potential of individual cells would be useful for cytological and biochemical analysis of in vitro aging.

We have previously reported that the extent of the adsorption of conA-coated RBCs to the surface of human diploid fibroblasts increased continuously throughout their lifespan and could serve as a marker of cellular aging which can be examined on individual cells [12-14]. In addition, we showed that in the mass culture system, small cells rapidly incorporating [³H]thymidine in late passage populations adsorbed RBCs well, unlike similar cells in early passage populations, resembling large slowly or non-[³H]thymidine-incorporating cells in the same populations with respect to this cell surface property [15]. In the present work, we examined the RBC adsorption capacity of individual clones in young and old cultures to determine its relation to colony size (thus proliferative property under the colony culture condition) and culture age.

Materials and Methods

Normal human diploid fibroblasts (TIG-1) were derived from the lung of a female fetus and were maintained in Minimum Essential Medium (MEM) supplemented with non-essential amino acids (Gibco, G-77) and with 10% fetal bovine serum (Gibco, I-76) as previously described [12]. For colony culture, low passage cells were plated on 21 cm² plastic dishes (Falcon) at a density of 100 cells per dish, and late passage cells at a density of 250 cells per dish. They were maintained for 2 weeks in the same medium supplemented with 20% fetal bovine serum. One or 2 days before the RBC adsorption assay, the medium was changed to minimize non-specific binding of conA-coated RBCs to the plastic substrate. Details of the assay procedures have been described in a previous report [12]. The number of adsorbed RBCs was counted on enlarged microscopic photographs taken following fixation with glutaraldehyde and staining with crystal violet. In colonies achieving five divisions